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Fluconazole Disk Diffusion Procedure for Determining Susceptibility of *Candida* Species

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A simple disk diffusion test was defined for quick determination of the susceptibility of *Candida* species to fluconazole. The standard macrotube dilution reference method, the fluconazole E test, and a 25-μg fluconazole disk test were all performed with each of 250 *Candida* species selected to provide a broad range of fluconazole MICs. All three methods were in excellent agreement. On RPMI 1640-glucose agar, isolates with inhibition zone diameters of ≥19 mm were all susceptible (MIC, ≤8.0 μg/ml) by the E test and 94% were susceptible by the macrotube method. Strains with smaller zones were either resistant, intermediate (dose-dependent susceptibility), or susceptible by the reference methods. The disk test did not adequately separate fully resistant strains from those with dose-dependent susceptibility: additional quantitative tests are needed for the few strains that are not unequivocally susceptible by the disk method.

With improved medical and surgical care, immunocompromised patients are now surviving longer, only to succumb to infections. Fungal infections are particularly important in those patients. Antifungal chemotherapy is selected primarily empirically, but in vitro susceptibility data are needed to help guide that selection. Isolates from individual patients are occasionally monitored to determine whether the selection of resistant variants might explain mycological persistence.

Methods for evaluating the susceptibility of yeasts to antifungal agents have been the subject of numerous studies during the last decade (2, 4, 5, 10, 12, 16). A standard reference procedure has been described by the National Committee for Clinical Laboratory Standards (NCCLS) (7). That reference procedure is a macrotube dilution technique which is too cumbersome for use in most clinical laboratories. A broth microdilution adaptation of that procedure has been found to be acceptable (3, 11, 16). Although microdilution tests can usually be read after 24 h, 48-h readings are specified for the NCCLS macrotube test (7). The E test (AB Biodisk, Solna, Sweden) is a proprietary test that has also been found to be capable of giving reliable results that can often be read after 24 h of incubation (2, 16).

All of the above procedures define the MICs of several antifungal agents. Those MICs are generally reproducible within a range of ±1 dilution interval (5, 8, 12). There are no universally accepted breakpoints for defining fluconazole-susceptible and fluconazole-resistant categories. The state of the art is such that there should be an intermediate buffer zone, probably including two doubling concentrations. A wide range of dosage options is available for fluconazole, and when treating infections due to strains in the intermediate category, higher doses of fluconazole may be appropriate. Such strains may be said to have dose-dependent susceptibility (S-DD) to stress the need for high-dose therapy. Strains for which the MICs are ≥64 μg/ml are assumed to be resistant to fluconazole, although some patients might respond to high-dose therapy.

For use in a clinical laboratory, a simplified disk diffusion test has some important advantages. For practical reasons, that disk technique should be similar to the disk procedure that is being used to test antibacterial agents. It would be more difficult to implement if greatly different technologies were needed. In this report, we define a disk diffusion procedure that embraces many of the features of the NCCLS reference test and does not deviate greatly from a standardized disk procedure that is widely used to test antibacterial agents. Only the medium and criteria for measuring zone diameters need to be changed. The current report describes an initial feasibility study designed to determine whether a disk diffusion procedure can be used to test fluconazole susceptibility. A 25-μg disk was selected for this evaluation because preliminary studies by Peter Troke (17) indicated that it was optimal for separating strains of *Candida* spp. with different levels of susceptibility to fluconazole.

MATERIALS AND METHODS

Reference methods. The NCCLS macrotube dilution procedure (7) was used as the standard method for evaluation of the other methods. Although MICs were read after 24 and 48 h, the 48-h MIC was used as the reference endpoint. Every disk test was accompanied by an E test strip applied to the same plate, thus providing another MIC for evaluation of the disk procedure. The E tests were also read after 24 and 48 h, although 48-h MICs were used for evaluation.

Fluconazole testing powder was obtained from Pfizer Central Research, Sandwich, England. For the macrotube dilution procedure, serial dilutions of fluconazole were prepared and 0.1-ml volumes were dispensed into plastic tubes (12 by 75 mm), which were then frozen until needed. Each tube received 1.0 ml of RPMI 1640 broth when it was inoculated, and that diluted the drug 11-fold.

Media. RPMI broth was obtained from Sigma Chemical Company (St. Louis, Mo.) and was prepared with L-glutamine and buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS) organic buffer. The agar version of RPMI was solidified with 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.), and the glucose content was increased to a final concentration of 2% (15).

Inoculum. The isolates were stored at room temperature in sterile water and subcultured onto Sabouraud dextrose agar for two daily transfers to ensure purity and viability. Approximately five isolated colonies were then suspended in sterile saline. After thorough mixing with a Vortex mixer, the turbidity of the suspension was adjusted to match that of a McFarland 0.5 turbidity standard. To expedite that critical step, a spectrophotometer was used (10). Dilutions of that inoculum suspension were prepared and quantitatively subcultured to confirm the actual number of CFU per milliliter. The tests were repeated if the inoculum was not within the range of 0.5×10^3 to 2.5×10^3 CFU/ml.

RPMI-glucose agar in a 15-cm-diameter petri plate was inoculated with a swab moistened in an inoculum suspension adjusted to match a McFarland 0.5 turbidity standard. One fluconazole E test strip (0.06 to 256 μg/ml) and one 25-μg fluconazole disk (Oxoid, Basingstoke, England) was applied to each inoculated plate.

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TABLE 1. In vitro activity of fluconazole against 250 *Candida* spp. selected for evaluation of disk tests

Species[a] (no. of isolates tested)	Test method[b]	Geometric mean MIC (μg/ml) and 95% CI[c] at:	
		24 h	48 h
C. albicans (100)	Broth	1.17 (1.13–1.20)	1.49 (1.45–1.53)
	E test	2.71 (2.67–2.75)	2.47 (2.43–2.52)
C. krusei (42)	Broth	25.6 (22.2–29.1)	48.3 (44.2–52.4)
	E test	52.5 (47.7–57.3)	82.0 (74.5–89.5)
C. guilliermondii (25)	Broth	16.0 (15.9–16.1)	28.75 (28.7–28.8)
	E test	9.71 (9.69–9.73)	12.58 (12.5–12.6)
C. parapsilosis (40)	Broth	0.466 (0.46–0.47)	0.856 (0.85–0.87)
	E test	0.616 (0.61–0.62)	0.917 (0.90–0.93)
C. tropicalis (40)	Broth	0.63 (0.62–0.64)	1.06 (1.03–1.05)
	E test	0.59 (0.58–0.60)	0.54 (0.53–0.55)

[a] In addition, three *C. kefyr* isolates were also tested; all MICs were 0.25 or 0.5 μg/ml.

[b] Broth is the NCCLS macrotube reference method done with RPMI 1640 broth. E tests were done with RPMI-glucose agar. Both tests were read after 24 and 48 h.

[c] CI, 95% confidence interval that is likely to include the "true" geometric mean MIC.

Incubation. All tests were incubated in ambient air at 35°C, and the results were recorded after 24 h and again after 48 h of incubation.

Endpoint determination. For the NCCLS reference test, the MIC was determined as the lowest concentration inhibiting at least 80% of the growth (4, 7). That was determined by comparing each tube to a 1:5 dilution of the growth control tube. For agar-based tests, inhibitory zones were measured at the point where there was a sharp decline in the amount of growth (approximately 80% inhibition). Similar guidelines were used for reading E tests according to the manufacturer's instructions. To simplify analyses of data, MICs recorded with the E test were rounded up to the next even $\log_2$ dilution interval.

Interpretive criteria. To define zone diameter interpretive criteria, we elected to apply conservative MIC breakpoints of ≤8.0 μg/ml for susceptibility and ≥64 μg/ml for resistance. The intermediate category thus included strains for which the MIC was 16 or 32 μg/ml. Their susceptibility is dose dependent; they are not fully susceptible, nor are they truly resistant. This broad intermediate category seems appropriate when one considers the wide range of dosage options available for fluconazole (6, 13, 14). These interpretive criteria have recently been accepted by the NCCLS subcommittee on antifungal susceptibility tests and will appear in its next publication as tentative criteria subject to review by all interested parties.

Microorganisms. A collection of 250 *Candida* spp. was selected to provide a wide range of fluconazole MICs. The isolates were kindly provided by C. Hitchcock, Pfizer Central Research, Sandwich, England. Included were 100 *Candida albicans*, 42 *C. krusei*, 40 *C. tropicalis*, 40 *C. parapsilosis*, 25 *C. guilliermondii*, and 3 *C. kefyr* isolates. *C.* (*Torulopsis*) *glabrata* and *Cryptococcus neoformans* were not considered in this phase of our studies. Quality control strains included *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258.

RESULTS

Reference tests. MICs were determined after 24 and 48 h with the NCCLS reference method and with the E test. Table 1 describes geometric mean MICs for each species group and for each method. The two methods produced essentially comparable MICs with a correlation coefficient of 0.89 for both 24- and 48-h MICs. At 48 h, 73% of the MICs were within a range of ±1 doubling concentration and 90% were within a range of ±2 doubling concentrations. The 48-h MICs for *C. krusei* were significantly greater than those read after 24 h with either method (Table 1). With *C. albicans*, 80% of the macrotube MICs and 90% of the E test MICs were the same (±1 doubling concentration) at 24 and 48 h. Some MICs increased, and others decreased, presumably because of difficulties in selecting endpoints after the first 24 h.

Disk susceptibility tests. The diameters of zones around 25-μg fluconazole disks were plotted against NCCLS reference test and E test MICs. Figures 1 and 2 demonstrate the excellent correlation between 48-h zone sizes and 48-h MICs. For both reference methods, regression statistics defined interpretive breakpoints of ≥19 mm for susceptibility (MIC, <16 μg/ml) and ≤14 mm for resistance (MIC, >32 μg/ml). Table 2 describes the interpretive agreements between different tests. Predictive values for a susceptible disk test result were improved slightly by incubating the test disks for a full 48 h. That involved tests with *Candida* spp. other than *C. albicans*, especially *C. krusei*. Predictive values for a resistant disk test result did not exceed 82%, primarily because of strains that were resistant by the disk test but intermediate (S-DD) by MIC categories. The disk test showed fewer discrepancies when compared to the agar-based E test rather than to the NCCLS reference broth dilution method.

Quality control. Our MIC control values were within the limits proposed by Pfaller et al. (8). Disk tests gave zones that fell into the following ranges for 10 to 12 determinations with each control strain: *C. albicans* ATCC 90028, 32 to 43 mm; *C. parapsilosis* ATCC 22019, 26 to 37 mm; *C. krusei* ATCC 6258, 6 to 17 mm.

DISCUSSION

To be easily integrated into the work flow of a clinical laboratory, an antifungal susceptibility test should be as close as possible to the method that is being used to test antibacterial

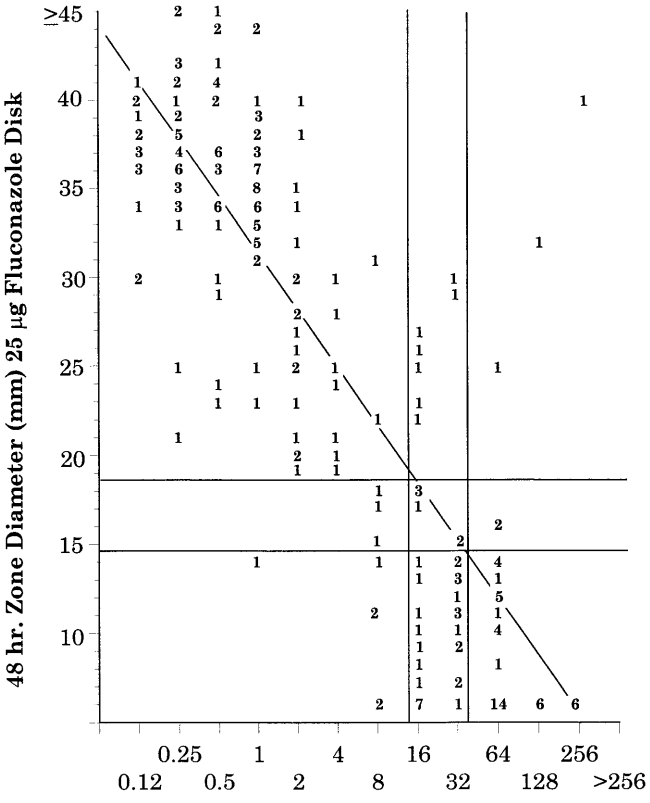

FIG. 1. Regression analysis correlating 48-h zones of inhibition around 25-μg fluconazole disks with 48-h MICs determined by the NCCLS macrotube dilution method (7). The regression statistic is $y = 60.4 - 3.20x$, $r = 0.84$.

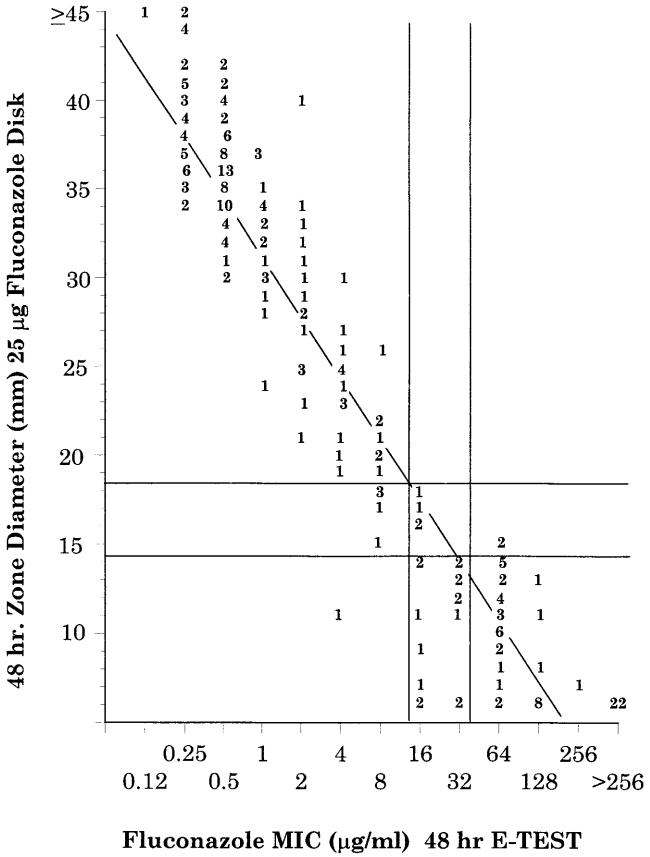

FIG. 2. Regression analysis correlating 48-h zones of inhibition around 25-μg fluconazole disks with 48-h fluconazole MICs determined by E tests. Both procedures were performed on the same plate of RPMI-glucose agar. The regression statistic is $y = 64.3 - 3.60x$, $r = 0.95$.

agents. The disk diffusion procedure that we propose differs from the standard method only in the use of RPMI-glucose agar rather than Mueller-Hinton or Iso-Sensitest agar. Plans are currently being made to evaluate the possibility of using simpler, less expensive agar media for the disk screening tests. For testing of yeasts, the inocula are adjusted by using a spectrophotometer to compare turbidity to that of a McFarland 0.5 standard. Bacterial inocula can be adjusted in the same way. Zone size limits for quality control strains have yet to be defined by a multilaboratory collaborative study. Intralaboratory reproducibility also needs to be determined with a wide range of *Candida* species and other yeasts. The subjectiveness of zone size measurements adds an important source of variability to the test. Zones of inhibition are defined in much the same way as sulfisoxazole or trimethoprim-sulfamethoxazole zones are read when testing bacteria. Selection of broth dilution MIC endpoints (80% inhibition) presents a similar source of variability that must be controlled, but that is not unique to tests of antifungal agents. Individuals doing such tests must first gather experience to be certain that they are all reading the tests in the same way. An initial learning exercise is very important before any results are reported.

With nearly all *Candida* species, disk tests can be read after the first 24 h of incubation: a second day is required if there is not a good lawn of growth on the first day. For species other than *C. albicans*, it may be prudent to use a full 48-h incubation routinely. That is particularly important for tests of *C. krusei*, since resistance is not always seen after the first 24 h. It is entirely possible that *C. krusei* could be assumed to be resistant to fluconazole without any in vitro tests.

Our disk test seems to be appropriate for testing of most *Candida* species but is not likely to be applicable to species that grow poorly on RPMI-glucose agar. Preliminary studies have shown relatively poor growth of *C.* (*Torulopsis*) *glabrata* and *C. neoformans* on the agar medium. The disk procedure is intended primarily for tests of *C. albicans.*

It is difficult to determine when a *Candida* sp. should be defined as clinically resistant to fluconazole because of the broad range of dosage options available to physicians. How-

TABLE 2. Predictive values of 24- and 48-h disk diffusion susceptibility tests compared to two reference methods, both read after 48 h of incubation

Reference method and MIC category	No. of strains classified by RPMI disk test					
	24-h			48-h		
	≥19 mm (susceptible)	15–18 mm (intermediate)	≤14 mm (resistant)	≥19 mm (susceptible)	15–18 mm (intermediate)	≤14 mm (resistant)
NCCLS broth dilution[a]						
Susceptible[b]	152	7	2	152	3	6
S-DD[c]	8	0	13	5	4	14
S-DD[d]	3	6	10	2	2	15
Resistant[e]	7	8	32	3	2	42
E test on RPMI agar[f]						
Susceptible[b]	163	2	1	162	5	1
S-DD[c]	3	3	5	0	4	7
S-DD[d]	1	4	4	0	0	9
Resistant[e]	3	12	47	0	2	60

[a] In comparison with the NCCLS test, the predictive value for a susceptible disk test result was 89% at 24 h and 94% at 48 h; predictive value for a resistant disk test result was 56% at 24 h and 55% at 48 h.
[b] MIC, ≤8.0 μg/ml.
[c] MIC, 16 μg/ml.
[d] MIC, 32 μg/ml.
[e] MIC, ≥64 μg/ml.
[f] In comparison with the E test on RPMI agar, the predictive value for a susceptible disk test result was 96% at 24 h and 100% at 48 h; predictive value for a resistant disk test result was 82% at 24 h and 78% at 48 h.

ever, it seems reasonable to assume that any strain for which the MIC is ≤8.0 μg/ml is susceptible clinically (1, 6, 13, 14), and over 90% of *C. albicans* isolates should fall into that category (9). With the disk test as a screening procedure, nearly all *C. albicans* isolates will be susceptible, and they can be reported as such without further testing. On the other hand, the few strains that are not susceptible by the disk test can be subjected to more quantitative procedures such as E tests or microdilution tests. With appropriate interpretation of the resulting MICs, one can separate strains with dose-dependent susceptibility (MIC of 16 or 32 μg/ml) from those that are resistant (MIC of ≥64 μg/ml). Dose-dependent susceptibility emphasizes the need for high-dose therapy for a favorable clinical response. The severity of the infection and the immune status of the patient are two other variables that determine the final outcome of chemotherapy (1, 6, 13, 14).

The disk test procedure outlined in this report provides encouraging results. Additional multilaboratory studies are needed to document inter- and intralaboratory reproducibility and to establish quality control parameters. In addition, consideration should be given to the possibility of using more potent disks to separate fully resistant strains from those with dose-dependent susceptibility. Additional studies with other antifungal agents are also needed to maximize the test's usefulness. In the interim, the current procedure might be of value to clinical laboratory workers who want to screen occasional isolates of *C. albicans* for susceptibility to fluconazole.

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